

TWIST1 Plays a Pleiotropic Role in Determining the Anaplastic Thyroid Cancer Phenotype

Paolo Salerno, Ginesa Garcia-Rostan, Sara Piccinin, Tammaro Claudio Bencivenga, Gennaro Di Maro, Claudio Doglioni, Fulvio Basolo, Roberta Maestro, Alfredo Fusco, Massimo Santoro, and Giuliana Salvatore

Dipartimento di Biologia e Patologia Cellulare e Molecolare c/o Istituto di Endocrinologia ed Oncologia Sperimentale del Consiglio Nazionale delle Ricerche (P.S., T.C.B., G.D.M., A.F., M.S.), Università di Napoli "Federico II," 80131 Naples, Italy; Institute of Molecular Pathology and Immunology (G.G.-R.), University of Porto, 4200-465 Porto, Portugal; Instituto de Biología y Genética Molecular (G.G.-R.), Universidad Valladolid–Consejo Superior de Investigaciones Científicas, 47003 Valladolid, Spain; Experimental Oncology 1 (S.P., R.M.), Centro di Riferimento Oncologico, Istituto di Ricovero e Cura a Carattere Scientifico, Aviano National Cancer Institute, 33081 Aviano (Pordenone), Italy; Università Vita-Salute San Raffaele (C.D.), Istituto Scientifico San Raffaele, 20132 Milan, Italy; Division of Pathology (F.B.), Department of Surgery, University of Pisa, 56126 Pisa, Italy; and Dipartimento di Studi delle Istituzioni e dei Sistemi Territoriali (G.S.), Università "Parthenope," 80133 Naples, Italy

Context: Anaplastic thyroid carcinoma (ATC) is one of the most aggressive human tumors; it is characterized by chemoresistance, local invasion, and distant metastases. ATC is invariably fatal.

Objective: The aim was to study the role of TWIST1, a basic helix-loop-helix transcription factor, in ATC.

Design: Expression of TWIST1 was studied by immunohistochemistry and real-time PCR in normal thyroids and well-differentiated, poorly differentiated, and ATC. The function of TWIST1 was studied by RNA interference in ATC cells and by ectopic expression in well-differentiated thyroid carcinoma cells.

Results: ATCs up-regulate TWIST1 with respect to normal thyroids as well as to poorly and well-differentiated thyroid carcinomas. Knockdown of TWIST1 by RNA interference in ATC cells reduced cell migration and invasion and increased sensitivity to apoptosis. The ectopic expression of TWIST1 in thyroid cells induced resistance to apoptosis and increased cell migration and invasion.

Conclusions: TWIST1 plays a key role in determining malignant features of the anaplastic phenotype *in vitro*. (*J Clin Endocrinol Metab* 96: E772–E781, 2011)

Thyroid neoplasms include a broad spectrum of histotypes, ranging from benign adenomas to differentiated papillary and follicular, poorly differentiated, and rapidly growing anaplastic carcinomas (1, 2). Papillary thyroid carcinoma (PTC) far outnumbers the other morphological subtypes and is characterized, in general, by an indolent phenotype with a 10-yr survival rate of up to 90% (1, 2). Poorly differentiated carcinomas (PDC) include a heterogeneous group of neoplasms with morphological

features and clinical characteristics intermediate between those of well-differentiated and anaplastic carcinomas (1, 2). Anaplastic thyroid carcinomas (ATC) represent less than 2% of all thyroid cancers but are responsible for more than 50% of thyroid cancer mortality, with a mean survival time from diagnosis of 4–12 months (3). ATC is highly invasive, and the majority of ATC patients die from suffocation due to locoregional disease extension or because of overwhelming distant metastatic disease. Surgical

treatment, radiotherapy, and chemotherapy, based primarily on doxorubicin and cisplatin, show little efficacy in ATC patients (3, 4). ATC cells feature a highly mitogenic and motile phenotype and epithelial-mesenchymal transition and are refractory to apoptotic cell death (3, 4). ATC features genetic lesions that are typical of a well-differentiated carcinoma, namely BRAF or RAS point mutations. Only a few genetic lesions have been identified exclusively in ATC, *i.e.* p53, PI3KCA, or β -catenin mutations (5–8). Therefore, the molecular mechanisms driving the establishment of the highly aggressive anaplastic phenotype are still largely unknown (2–5).

We have recently identified, through a cDNA microarray analysis, a gene expression signature that is associated with the highly proliferative and aneuploid ATC phenotype (9). Among the genes highly up-regulated in ATC *vs.* normal tissue and PTC, we have isolated TWIST1. TWIST1 is a highly conserved basic helix-loop-helix transcription factor that plays a key role in mesodermal, myoblast, and osteoblast differentiation (10, 11). Mutational inactivation of TWIST1 is responsible for the Saethre-Chotzen syndrome, an autosomal dominant disorder characterized by premature fusion of the cranial sutures, skull deformations, limb abnormalities, and facial dysmorphism (12). TWIST1 plays an important role in the development and progression of human cancer. TWIST1 overexpression is reported in many human tumors, including rhabdomyosarcoma, glioma, melanoma, breast, gastric, and prostate carcinomas (13, 14). Elevated TWIST1 protein levels are associated with advanced tumor stage and poor prognosis in several cancer types (14, 15). TWIST1 promotes epithelial-mesenchymal transition (16, 17). TWIST1 gene amplification is associated with resistance to chemotherapeutic agents (18, 19). Finally, TWIST1 inhibits premature senescence in cancer cells (20).

Here we report that TWIST1 plays a key role in the ATC phenotype *in vitro* and suggest that it may mediate chemoresistance of ATC cells.

Materials and Methods

Reagents

Staurosporine and cisplatin were obtained from Sigma-Aldrich (St. Louis, MO).

Cell cultures

Human cell lines (S11N, P5 4N, 8505C, CAL62, SW1736, OCUT-2, ACT-1, TPC-1, BCPAP) (21–23), rat cell lines (PC RET/PTC1, PC RET/PTC3, PC *v*-HRAS, PC-BRAF-V600E, PC-TRK-T1, PC *v*-RAF, PC *v*-MOS, PC E1A, and PC E1A-*v*-RAF) (24, 25), and culture conditions are detailed in the Supplemental Data (published on The Endocrine Society's Journals Online web site at <http://jcem.endojournals.org>).

Tissue samples

Tumors and normal thyroid (NT) tissue samples for immunohistochemical analysis were retrieved from the files of the Pathology Department of the Hospital Central de Asturias (Oviedo University, Asturias, Spain) and of the Hospital Clinico Universitario de Santiago de Compostela (Santiago de Compostela University, Galicia, Spain). Tumors and NT tissue samples for RNA extraction and quantitative RT-PCR were retrieved from the files of the Department of Surgery, University of Pisa (Pisa, Italy). Case selection was based on the histological findings and on the availability of adequate material for RNA extraction. All histological diagnoses were reviewed by two blinded pathologists (G.G.-R. and C.D.) according to the latest recommendations about diagnostic features of PTC, PDC, and ATC (26–28). PDC were defined as malignant tumors of follicular cells displaying predominant solid/trabecular/insular growth patterns, high-grade features such as mitoses (more than three to five mitoses \times 10 high power field) and/or necrosis and convoluted nuclei, with or without concurrent differentiated components of the follicular or papillary type. ATC were defined as tumors displaying admixtures of spindle, pleomorphic giant, and epithelioid cells; high mitotic activity; extensive coagulative necrosis with irregular borders; and infiltration of vascular walls often accompanied by obliteration of the vascular lumina. After microscopic examination of exhaustively sampled specimens, 32 tumors were classified as PTC, 93 as PDC, and 56 as ATC. Processing of samples and of patient information proceeded in agreement with review board-approved protocols.

Immunohistochemistry

Formalin-fixed and paraffin-embedded 3- to 5- μ m-thick tumor sections were deparaffinized, placed in a solution of absolute methanol and 0.3% hydrogen peroxide for 30 min, and treated with blocking serum for 20 min. The slides were incubated with mouse monoclonal antibodies against TWIST1 (sc-81417; Santa Cruz Biotechnology, Santa Cruz, CA) and processed according to standard procedures. Negative controls by omitting the primary antibody were included in the assay. Cases were scored as positive when unequivocal brown staining was observed in the nuclei of tumor cells. Immunoreactivity was expressed as the percentage of positively stained target cells in four intensity categories (–, no staining; +, low/weak; ++, moderate/distinct; +++, high/intense). Twist1 score values were independently assigned by two blinded investigators (G.G.-R. and C.D.), and a consensus was reached on all scores used for computation.

RNA extraction and expression studies

Total RNA was isolated with the RNeasy Kit (QIAGEN, Crawley, West Sussex, UK). The quality of the RNAs was verified by the 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany); only samples with an RNA integrity number value above 7 were used for further analysis. Real-time PCR was performed as detailed in the Supplemental Data: for the calculation of expression fold changes, sample 1 represented each single tumor sample, and sample 2 was the average of all ($n = 22$) NTs. Microarray methods are also reported in the Supplemental Data.

Protein studies

Immunoblotting was carried out according to standard procedures. Anti-TWIST1 (sc-81417) and anti-p53 (Pab 240) monoclonal antibodies were from Santa Cruz Biotechnology; monoclonal anti- α -tubulin antibody was from Sigma-Aldrich; anti-cleaved (Asp175) caspase-3 p17 and p19 fragments polyclonal (5A1) antibody was from Cell Signaling Technology, Inc. (Beverly, MA). Secondary antimouse and antirabbit antibodies coupled to horseradish peroxidase were from Santa Cruz Biotechnology.

RNA silencing

Small inhibitor duplex RNA targeting TWIST1 (no. 3, 5, and 7) and the scrambled control [nonspecific small interfering RNA (siRNA) duplex containing the same nucleotides but in irregular sequence] have been described previously (16) and were chemically synthesized by Sigma-Aldrich. Based on its higher silencing efficiency, TWIST1 siRNA 3 (hereafter referred to as TWIST1 siRNA) was selected and used throughout the paper. The day of transfection, 1×10^5 cells were incubated with 50 nM siRNA and electroporated using MicroPorator (MP-100, Digital Bio; Euroclone, Milan, Italy) according to the manufacturer's instructions. Cells were harvested 24, 48, and 72 h after transfection, counted, and analyzed for protein expression. Methods used to determine cell viability, motility, and invasion are detailed in the Supplemental Data.

TWIST1 transfection

The pcDNA 3-TWIST1 vector is described elsewhere (29). TPC-1 cells were transfected by using the Lipofectamine Reagent (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. Two days later, G418 (Invitrogen) was added at a concentration of 1.2 mg/ml. Two mass populations of several clones and three independent cell clones were isolated, expanded, and screened for TWIST1 expression by Western blot and RT-PCR analysis. One mass population and two cell clones transfected with the control pcDNA 3 vector were expanded. To generate stable shRNA (short hairpin RNA)-expressing cell line, CAL62 cells were transfected with shTWIST1 and shLUC vectors (20) by using the Lipofectamine reagent (Invitrogen) according to the instructions of the manufacturer. Two days later, puromycin (Invitrogen) was added at a concentration of 0.5 mg/ml. Mass populations and several cell clones were isolated, expanded, and screened for TWIST1 knockdown by Western blot and RT-PCR analysis. Methods used to determine cell viability, motility, and invasion are detailed in the Supplemental Data.

Statistical analysis

Statistical analyses were carried out using the GraphPad In-Stat software program (version 3.06.3; GraphPad Software, Inc., San Diego, CA). All *P* values were two-sided, and differences were significant when *P* < 0.05.

Results

Up-regulation of TWIST1 in ATC

We evaluated TWIST1 expression levels by immunohistochemistry in 157 human tissues including: 15 NT, 13

PTC, 88 PDC, and 41 ATC samples. Representative immunohistochemical staining is shown in Fig. 1A, and the entire dataset is reported in Table 1. TWIST1 was virtually undetectable in NT, PTC, and PDC samples. In contrast, 49% of the ATC samples (20 of 41) were positive for TWIST1 expression. Positivity ranged between at least 5 and no more than 25% (+) to at least 60% (+++) of cells (Fig. 1A and Table 1); no staining was observed in the absence of the primary antibody (data not shown). TWIST1 positivity correlated with moderate/high proliferation rate assessed by Ki67/MIB1 immunoreactivity (Freeman-Halton extension of the Fisher exact probability test, *P* = 0.048) (Supplemental Table 1). There was also a trend for a significant association with up-regulation of the cell cycle regulated minichromosome maintenance 5 protein (Freeman-Halton extension of the Fisher exact probability test, *P* = 0.11) (data not shown). TWIST1 expression correlated with the fusocellular ATC phenotype and inversely correlated with the epithelioid ATC phenotype (χ^2 test, *P* = 0.0002; Freeman-Halton extension of the Fisher exact probability test, *P* = 0.0001) (Supplemental Table 1), suggesting that TWIST1 is involved in mesenchymal transition of ATC cells. Accordingly, there was a trend toward a correlation between TWIST1 up-regulation and lack of β -catenin staining at the plasma membrane (Freeman-Halton extension of the Fisher exact probability test, *P* = 0.119) (data not shown). Finally, TWIST1 positivity correlated with p53 positivity (Freeman-Halton extension of the Fisher exact probability test, *P* = 0.030) (Supplemental Table 1).

To determine whether TWIST1 up-regulation also occurred at the RNA level, we examined an independent set of ATC (*n* = 15), PDC (*n* = 4), PTC (*n* = 19), and NT (*n* = 22) samples by quantitative RT-PCR. As shown in Fig. 1B, TWIST1 mRNA was up-regulated by more than 5-fold in about 50% (seven of 15) of the ATC samples, with values greater than 10-fold in 13% (two of 15) of them. NT, PTC, and PDC samples expressed lower TWIST1 levels compared with ATC (*P* < 0.001) (Fig. 1B).

In vertebrates, there are two TWIST genes, TWIST1 and TWIST2 (also known as Dermo1), and their encoded proteins show an identity in the basic helix-loop-helix domain of more than 90% (20). Therefore, we also measured TWIST2 expression by quantitative RT-PCR in thyroid tissue samples. TWIST2 was overexpressed in some cases, but at a lower extent with respect to TWIST1 (Supplemental Fig. 1). Indeed, TWIST2 was up-regulated by more than 2-fold in only about half of the ATC samples (four of nine), with values greater than 5-fold in only one of nine ATC samples. No PTC sample up-regulated TWIST2 (Supplemental Fig. 1A).

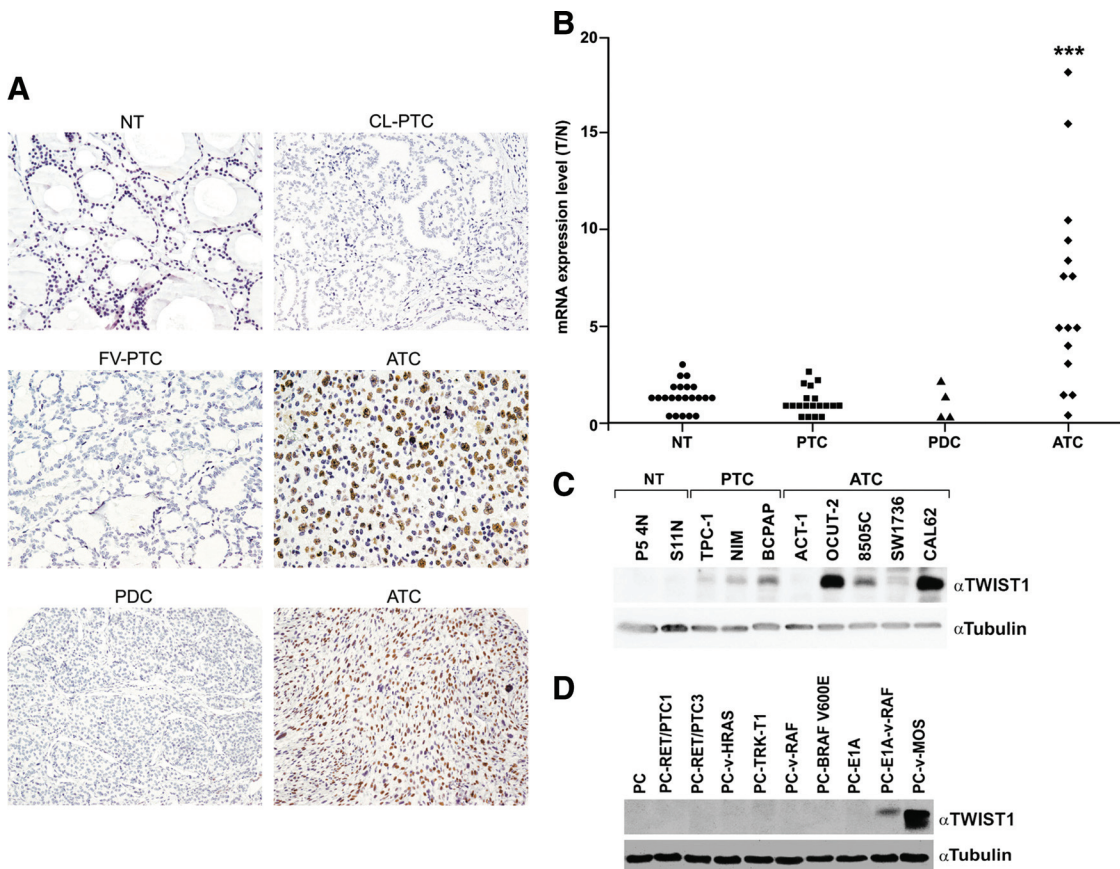


FIG. 1. Expression of TWIST1 in thyroid tissue samples and in cell lines. A, Immunohistochemical analysis of TWIST1 protein expression in normal and malignant thyroid tissues. Representative histological sections from NT (20× magnification), classical PTC (CL-PTC; 10× magnification), follicular variant PTC (FV-PTC; 20× magnification), PDC (4× magnification), and ATC (20× and 4× magnification) stained with a mouse monoclonal anti-TWIST1 antibody are shown. The NT, CL-PTC, FV-PTC, and PDC sections were negative for TWIST1, whereas the two ATC cases featured high/intense immunoreactivity levels (+++, e.g. ≥60% of cells); in particular, the ATC sample at 20× magnification showed 65% of positive cells, whereas the ATC at 4× magnification showed 85% of positive cells. B, Quantitative RT-PCR of TWIST1 mRNA in NT (n = 22), PTC (n = 19), PDC (n = 4) and ATC (n = 15) snap-frozen tissue samples. The level of TWIST1 expression in each sample was measured by comparing its fluorescence threshold with the average fluorescence threshold of the NT samples. The average results of triplicate samples are plotted. C, NT follicular cells (P5 4N and S11N), PTC (TPC-1, NIM, and BCPAP), and ATC (ACT-1, OCUT-2, 8505C, SW1736, and CAL62) cell lines were analyzed by immunoblot using a mouse monoclonal anti-TWIST1 antibody. Anti α -tubulin monoclonal antibody was used as a control for equal protein loading. D, Immunoblot of TWIST1 expression in rat thyroid PC cells expressing the indicated oncogenes. ***, $P < 0.001$.

Up-regulation of TWIST1 in thyroid cancer cell lines

We analyzed TWIST1 expression in cultured human thyroid cells. To this aim, we used primary cultures of NT follicular cells (P5 4N and S11N) and a panel of PTC

TABLE 1. TWIST1 expression in thyroid samples (n = 157)

Tissue	TWIST1 positivity, % of positive samples (positive/total samples)		
NT	0% (0/15)		
PTC	0% (0/13)		
PDC	0% (0/88)		
ATC	49% (20/41)	+	17% (7/41)
		++	15% (6/41)
		+++	17% (7/41)

+, ≥5 to ≤25% of positive cells; ++, >25 to <60% of positive cells; +++, ≥60% of positive cells.

(TPC-1, NIM, BCPAP) and ATC (ACT-1, OCUT-2, 8505C, SW1736, CAL62) cell lines. Western blot analysis showed up-regulation of a band at approximately 26 kDa, which corresponded to the TWIST1 protein only in the ATC cell lines OCUT-2, 8505C, and CAL62 (Fig. 1C). TWIST1 expression was lower in the other ATC cells and in all the PTC cell lines analyzed, whereas it was undetectable in NT cells (Fig. 1C). RT-PCR analysis confirmed the Western blot results (data not shown). Finally, CAL62 and BCPAP also up-regulated TWIST2 mRNA by more than 2-fold with respect to NT cells (Supplemental Fig. 1B).

To confirm TWIST1 expression in a model cell system and to start exploring whether TWIST1 up-regulation correlated with loss of differentiation or with an aggressive tumor phenotype, we used a panel of rat thyroid follicular Fischer rat-derived thyroid follicular cell

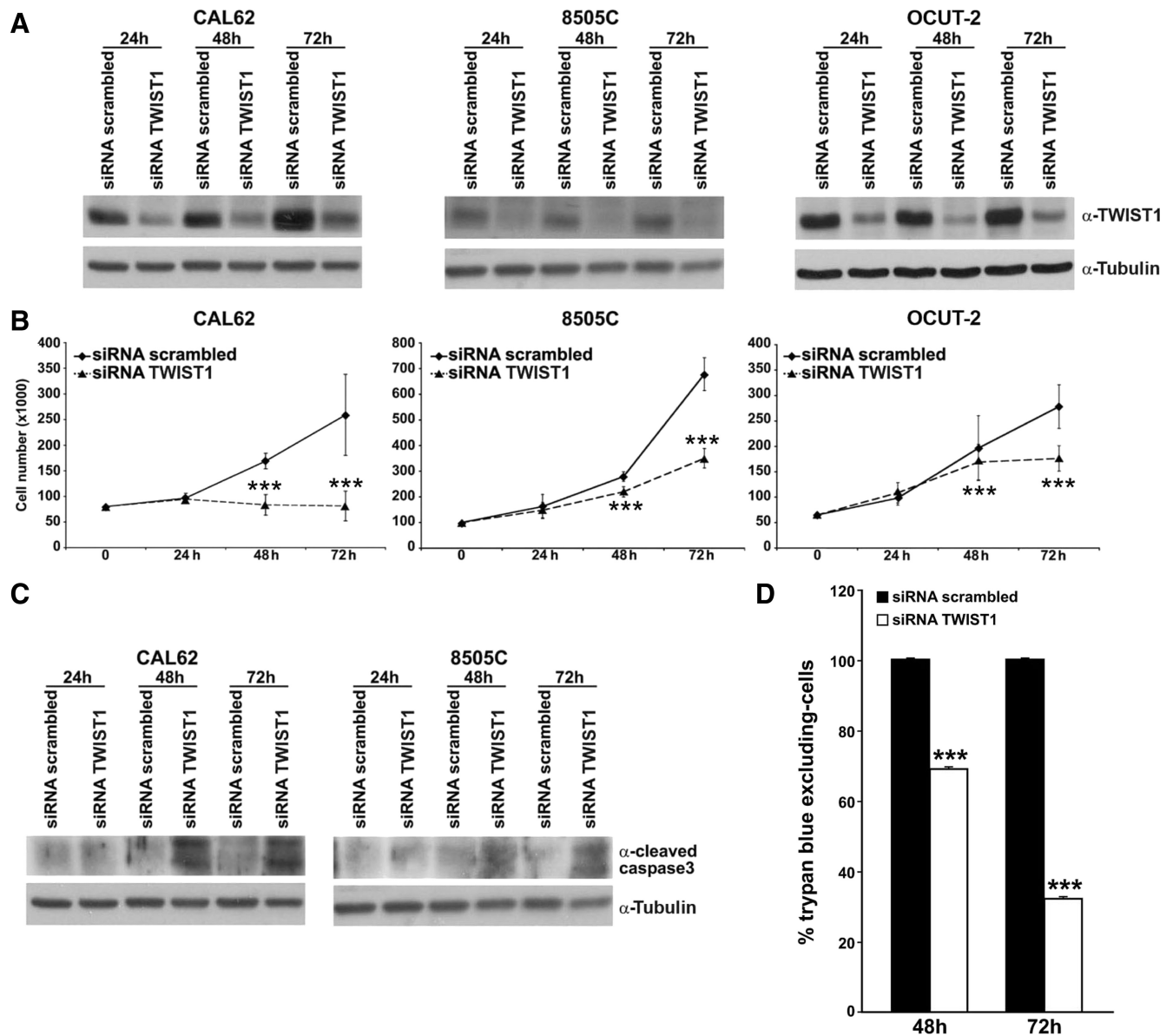


FIG. 2. Effects of TWIST1 knockdown in ATC cells. A, CAL62, 8505C, and OCUT-2 cells were transfected with TWIST1 siRNA or with scrambled siRNA. Cells were harvested at different time points, and protein lysates were subjected to immunoblotting with the indicated antibodies. B, CAL62, 8505C, and OCUT-2 cells were transfected with TWIST1 siRNA or with scrambled siRNA and counted at different time points. Values represent the average of triplicate experiments \pm 95% confidence intervals. C, CAL62 and 8505C cells were transfected with TWIST1 siRNA or with scrambled siRNA. Cells were harvested at different time points, and protein lysates were subjected to immunoblotting with the indicated antibodies. D, The indicated cell lines were transfected with TWIST1 siRNA or scrambled siRNA; after 48 and 72 h, cells were collected by trypsinization, stained for 10 min with trypan blue, and counted in triplicate. The percentage of trypan blue excluding cells compared with cells transfected with siRNA scrambled is reported \pm sd. ***, $P < 0.001$.

line PC CI 3 (PC) cells adoptively expressing various oncogenes (PC-RET/PTC1, PC-RET/PTC3, PC-v-HRAS, PC-TRK-T1, PC-v-RAF, PC-BRAF V600E, PC-E1A, PC-E1A-v-RAF, and PC-v-MOS). Although the expression of v-MOS and of the E1A/v-RAF combination enabled PC cells to grow in semisolid medium and to induce tumors in athymic mice, the expression of the RET/PTC1/3, HRAS, TRK, RAF (v-RAF and BRAF) and E1A oncogenes only caused loss of differentiation without fostering a tumorigenic phenotype (24, 25). Figure 1D shows that TWIST1 was only expressed in the PC cells

transformed by v-MOS, and at lower levels by E1A + v-RAF. Therefore, TWIST1 up-regulation correlated with malignant phenotype rather than loss of differentiation of rat thyroid cells.

Knockdown of TWIST1 induces apoptosis of ATC cells

We evaluated the effects of TWIST1 ablation in ATC cells by RNA interference. We initially tested, by Western blot in CAL62 cells, the efficiency of TWIST1 ablation using three different siRNA (no. 3, 5, and 7) (16). TWIST1

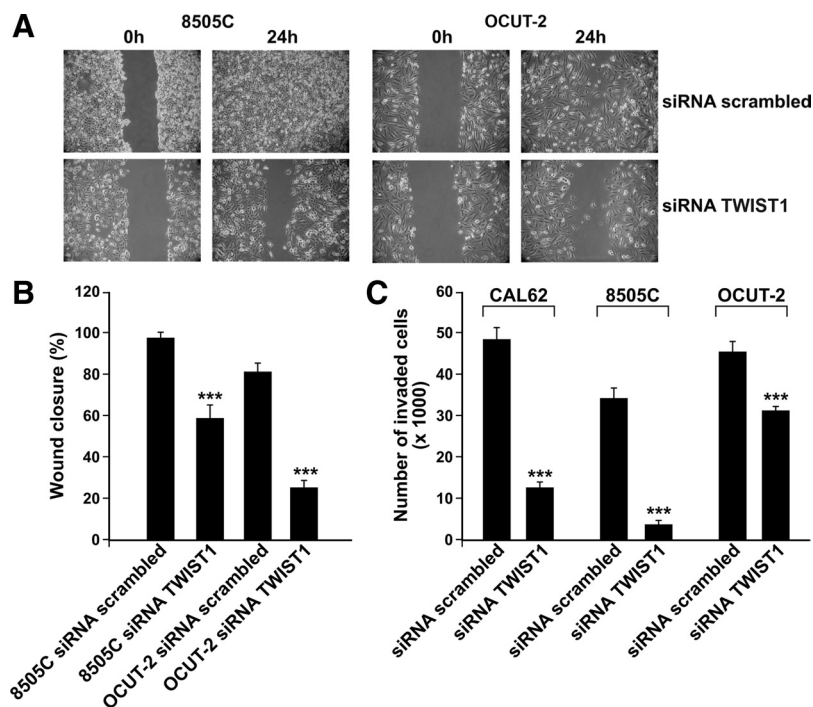


FIG. 3. Effects of TWIST1 knockdown on ATC cell migration and invasion. A, Cells were transfected with TWIST1 siRNA or scrambled siRNA; a scraped wound was introduced, and cell migration into the wound was monitored at 24 h. B, Wound closure was measured by calculating pixel densities in the wound area and expressed as percentage of wound closure of triplicate areas \pm SD. C, Cells were transfected with TWIST1 siRNA or scrambled siRNA; after transfection, cells were seeded in the upper chamber of transwells and incubated for 12 h; the upper surface of the filter was wiped clean, and cells on the lower surface were stained and counted. Invasive ability was expressed as number of invaded cells. Values represent the average of triplicate experiments \pm SD. ***, $P < 0.001$.

siRNA 3 (hereafter named TWIST1 siRNA) reduced TWIST1 protein levels of about 60% (Fig. 2A) and therefore was selected for further experiments, whereas the other two siRNAs (5 and 7) were less effective, with siRNA 5 being practically devoid of any effect and siRNA 7 depleting TWIST1 protein by less than 30% (Supplemental Fig. 2).

We knocked down TWIST1 by transient siRNA TWIST1 transfection in CAL62, 8505C, and OCUT-2 cells. As shown in Fig. 2A, a TWIST siRNA silenced the TWIST1 protein starting at 24 h after transfection, and the effect lasted up to 72 h, whereas a scrambled siRNA control had no effect (Fig. 2A). Thus, cells were transfected with TWIST1 siRNA or with scrambled siRNA and counted at different time points (24, 48, and 72 h) (Fig. 2B). Seventy-two hours after transfection, CAL62 cells transfected with scrambled siRNA numbered 259×10^3 , whereas those transfected with TWIST1 siRNA numbered 81×10^3 ($P = 0.0008$); 8505C cells transfected with scrambled siRNA numbered 679×10^3 , whereas those transfected with TWIST1 siRNA numbered 351×10^3 ($P < 0.0001$); OCUT-2 cells transfected with scrambled siRNA numbered 278×10^3 , and those transfected with

TWIST1 siRNA numbered 176×10^3 ($P = 0.0009$) (Fig. 2B). It should be noted that CAL62, but not 8505C and OCUT-2, expressed detectable levels of TWIST2 (Supplemental Fig. 1B). Thus, it is feasible, because of the high degree of homology between TWIST1 and TWIST2, that the effects of TWIST1 siRNA observed in CAL62 cells were due to the combined inhibition of TWIST1 and TWIST2. Accordingly, at 24 h after siRNA transfection, TWIST2 mRNA was down-regulated by 1.8-fold in CAL62 (data not shown).

At 48 and 72 h, siRNA TWIST1 induced cell apoptosis of CAL62 and 8505C cells as measured by immunoblot with an antibody for the cleaved products of caspase 3 (Fig. 2C). Accordingly, the percentages of trypan blue excluding (viable) cells, of CAL62 transfected with TWIST1 siRNA 48 and 72 h after transfection, were of 69 and 32%, respectively, with respect to scrambled control, confirming that TWIST1 depletion reduced thyroid cancer cell viability ($P < 0.001$) (Fig. 2D).

Because TWIST1 has been associated with premature senescence of cancer cells (20), we performed a senescence-associated β -galactosidase (SA- β -gal) staining assay on siRNA TWIST1-treated cells. Seventy-two hours after transfection with TWIST1 siRNA, the percentage of SA- β -gal-positive cells was 1.3%, whereas it was 0.7% in scrambled siRNA transfected cells. As a positive control, the percentage of SA- β -gal-positive cells was 38% in normal human diploid fibroblasts treated with Etoposide (Sigma-Aldrich) (data not shown). Thus, although significant, senescence induced by TWIST1 knockdown involved only a minor fraction of ATC cells.

Knockdown of TWIST1 impairs cell migration and invasion of ATC cells

We evaluated the migration (by a wound-healing assay) and invasion (by a Matrigel invasion assay) ability of TWIST1 siRNA-transfected cells compared with scrambled siRNA-transfected cells. As shown in Fig. 3A, 8505C and OCUT-2 cells transfected with the scrambled control efficiently migrated into the wound; in contrast, cells transfected with TWIST1 siRNA had a greatly reduced migrating ability ($P < 0.001$). Furthermore, cells transfected with TWIST1 siRNA had a reduced ability to invade Matrigel compared with control cells ($P < 0.001$) (Fig. 3B).

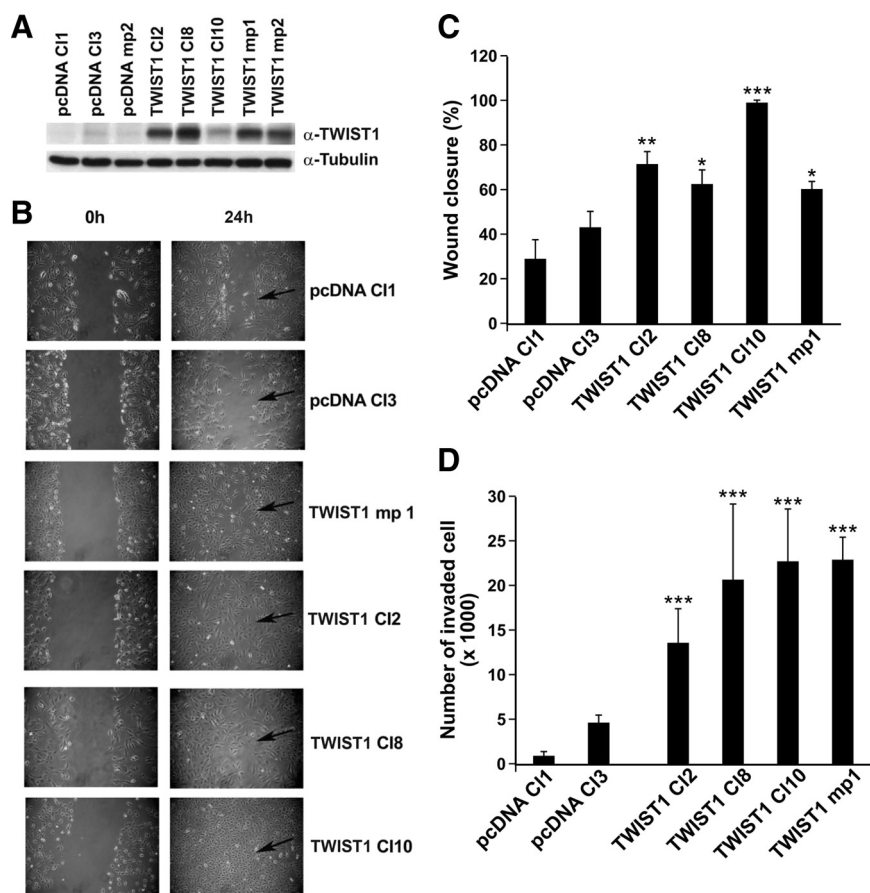


FIG. 4. Effects of TWIST1 overexpression on TPC-1 cell migration and invasion. A, Expression levels of TWIST1 in TPC-1-transfected cells. After G418 selection, cells were lysed and blotted with the indicated antibodies. B, A scraped wound was placed on the confluent monolayer of TPC-1 transfected with TWIST1 or the empty vector, and the cell migration into the wound was monitored at 24 h. Arrows indicate the site of wound closure. C, Wound closure was measured by calculating pixel densities in the wound area and expressed as percentage of wound closure of triplicate areas \pm SD. D, Cells were seeded in the upper chamber of transwells and incubated for 12 h; the upper surface of the filter was wiped clean, and cells on the lower surface were stained and counted. Invasive ability was expressed as number of invaded cells. Values represent the average of triplicate experiments \pm SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

Effects of stable silencing of TWIST1 in CAL62 cells

We stably transfected CAL62 cells with an shTWIST1 plasmid or with shLUC control (20). After antibiotic selection, cells were screened by Western blot for TWIST1 expression. A mass population (mp) (shTWIST1 mp) with a TWIST1 knockdown of approximately 52% was used for further study (Supplemental Fig. 3A). Consistent with data obtained upon transient TWIST1 silencing (Fig. 2), shTWIST1 mp cells showed decreased migration and invasion ability with respect to shLUC-transfected cells ($P < 0.001$) (Supplemental Fig. 3, B–D). To address effects of TWIST1 knockdown on chemosensitivity, cells were treated with cisplatin (200 or 1000 nM) or staurosporine (300 or 500 nM) and counted at 24 h. Upon treatment with cisplatin (1000 nM) or staurosporine (300–500 nM), shTWIST1 mp cells had a decreased viability compared with control cells ($P < 0.001$) (Supplemental Fig. 3E).

Finally, the number of colonies formed in semisolid medium (soft agar) was also reduced by 2-fold compared with the shLUC mp control ($P < 0.05$) (Supplemental Fig. 3F). Thus, TWIST1 ablation *in vitro* affected several hallmarks of malignancy of CAL62 cells, including anchorage-independent proliferation, survival, and invasion.

Ectopic TWIST1 promotes cell migration and invasion of PTC cells

PTC cells, TPC-1, which have low endogenous levels of TWIST1, were transfected with a TWIST1-expressing plasmid (pcDNA-TWIST1) or with the empty vector (pcDNA). Mass populations and cell clones were selected in G418 (1.2 mg/ml). TWIST1 expression was increased (~4- to 13-fold) in all the cell lines transfected with TWIST1 compared with the controls (Fig. 4A). Growth rate was similar in TWIST1- and vector-transfected control cell lines (data not shown). Therefore, we studied cell migration using the wound closure assay. As shown in Fig. 4, B and C, migration rate was higher in TWIST1-transfected cells than in control cells ($P < 0.05$). We next seeded TWIST1-transfected and control cells into the top chamber of transwells and evaluated their ability to invade Matrigel.

TPC-1 cells had basal levels of invasiveness, and TWIST1 overexpression further increased this ability by 4- to 6-fold ($P < 0.001$) (Fig. 4D). Thus, TWIST1 stimulated cell motility and invasion, although wound closure and Matrigel invasion extent were not directly proportional to the TWIST1 expression levels (Fig. 4). These findings suggest that the TWIST1 expression level is not the only molecular determinant of thyroid cancer cell invasive phenotype.

Ectopic TWIST1 protects PTC cells from apoptosis

We treated TPC-1 cells transfected with TWIST1 or control vector with different concentrations of cisplatin (200, 1000, and 2000 nM) and counted cell number at 24 h. As shown in Fig. 5A, cell viability was higher in TWIST1-transfected than in control cells upon treatment with the highest drug dose ($P < 0.05$). Moreover,

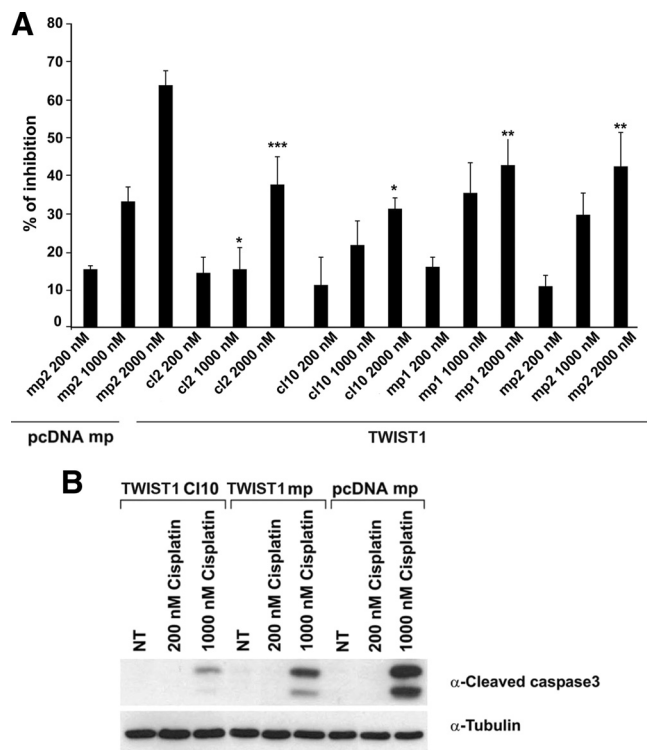


FIG. 5. Effects of TWIST1 overexpression on TPC-1 cell apoptosis. A, The cells were treated with increasing doses of cisplatin and counted 24 h after treatment. Data are shown as percentage of inhibition of cell viability. Values represent the average of three independent experiments \pm SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. B, The indicated cell lines were treated with increasing doses of cisplatin, lysed, and blotted with the indicated antibodies.

the amount of caspase 3 cleaved product was lower in TWIST1-transfected than in control cells (Fig. 5B).

Finally, we searched an Affymetrix microarray-based data set for genes that were previously reported to be TWIST1 targets and related to cell cycle and apoptosis control (30). TWIST1-overexpressing cells (TPC-1 TWIST1 mp1, TWIST1 mp2, and TWIST1 Cl2) up-regulated ($P < 0.05$), with respect to pcDNAmp control, the expression of AKT2 (average fold change \pm SD, 2 ± 0.5) and BCL-2 (average fold change \pm SD, 1.67 ± 0.2), whereas they down-regulated the expression of p21CIP/WAF1 (average fold change \pm SD, 0.7 ± 0.3). TWIST1 overexpression, instead, did not significantly change the expression of p14ARF, BAX, and TIMP1 in our system (data not shown).

Discussion

Thyroid cancer includes tumor types as different as well-differentiated carcinomas that have a very good prognosis and undifferentiated carcinomas or ATC that are among the most aggressive human cancers. As yet, the molecular players sustaining such different behavior are largely un-

known. Here, we demonstrate that TWIST1 is up-regulated in ATC samples. Overall TWIST1 up-regulation in ATC is more prominent at the RNA than at the protein level. It is possible that TWIST1 overexpression is affected not only at the transcriptional level but also at the post-transcriptional level. TWIST1 up-regulation was associated with mitotic index, as determined by Ki67/MIB1. However, the fact that TWIST1 did not influence cell proliferation in cultured cells suggests that it is not directly involved in controlling cell proliferation. In ATC samples, TWIST1 positivity also correlated with markers of mesenchymal transition (fusocellular phenotype) and malignancy (p53 positivity); however, correlation with p53 expression was not demonstrated in cultured thyroid cancer cells because TWIST1-positive (OCUT-2, 8505C, and CAL62) and TWIST1-negative (TPC-1, BCPAP, NIM, ACT-1, SW1736) cells both had either high (8505C, ACT-1) or low/undetectable (CAL62, OCUT-2, TPC-1, BCPAP, NIM, SW1736) p53 expression (Supplemental Fig. 4).

In vitro cellular models confirmed the role of TWIST1 in determining the ATC phenotype and identified the ATC features that were sustained by TWIST1 up-regulation. TWIST1 up-regulation did not correlate with loss of differentiation; indeed, it did not occur in PC cells that lost differentiation secondary to the expression of various oncogenes. Rather, TWIST1 overexpression correlated with a malignant phenotype being present in tumorigenic PC-v-MOS and PC-E1A+v-RAF cells (24). Moreover, TWIST1 expression was necessary to counteract spontaneous ATC cell apoptosis and to sustain the invasive and motile phenotype of ATC cells. Accordingly, when overexpressed in PTC cells, TWIST1 promoted cell migration and protected cells from apoptosis. Given the high homology between TWIST1 and -2, the TWIST siRNA also targeted TWIST2 (1.8-fold), albeit at lower levels than TWIST1 (2.5-fold). Thus, it is possible that at least in CAL62 cells effects were due to the combined inhibition of TWIST1 and TWIST2.

TWIST1 expression is responsive to Wnt-1 (31), IGF-I (32), and nuclear factor κ B (NF- κ B) signaling (33). Elements of the Wnt pathway, particularly CTNNB1 (the gene coding β -catenin), were found to be mutated in PDC and ATC (34). Moreover, NF- κ B is activated in human thyroid cancer cells, in particular in ATC (35). Therefore, both the Wnt-1/ β -catenin and NF- κ B pathways are good candidates as mediators of TWIST1 up-regulation in ATC.

Up-regulation of TWIST1 is associated with cellular resistance to anticancer drugs such as cisplatin, taxol, and vincristine in various types of cancers (18, 19, 36). Here, we show that TWIST1 overexpression protected thyroid

cancer cells from cell death induced by cisplatin and staurosporine. This suggests that TWIST1 may be exploited as a molecular marker of the response of thyroid cancer to chemotherapy.

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Address all correspondence and requests for reprints to: Giuliana Salvatore, Dipartimento di Studi delle Istituzioni e dei Sistemi Territoriali, Università “Parthenope,” Via Medina 40, 80133 Naples, Italy. E-mail: giuliana.salvatore@uniparthenope.it.

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